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Preparation, characterisation and maintenance of drug efficacy of doxorubicin-loaded human serum albumin (HSA) nanoparticles

S. Dreis [a], F. Rothweiler [b], M. Michaelis [b], J. Cinatl Jr. [b], J. Kreuter [a], K. Langer [a,*]

[a] *Institute of Pharmaceutical Technology, Biocenter Niederursel, Johann Wolfgang Goethe-University, Max-von-Laue-Straße 9, D-60438 Frankfurt/Main, Germany*
[b] *Institute of Medical Virology, University Hospital Medical School, Johann Wolfgang Goethe-University, Paul Ehrlich-Straße 40, D-60596 Frankfurt/Main, Germany*

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Abstract

Human serum albumin (HSA) nanoparticles represent promising drug carrier systems. Binding of cytostatics to HSA nanoparticles may diminish their toxicity, optimise their body distribution and/or may overcome multidrug resistance. In the present study, doxorubicin-loaded HSA nanoparticle preparations were prepared. Doxorubicin was loaded to the HSA nanoparticles either by adsorption to the nanoparticles' surfaces or by incorporation into the particle matrix. Both loading strategies resulted in HSA nanoparticles of a size range between 150 nm and 500 nm with a loading efficiency of 70–95%. The influence on cell viability of the resulting nanoparticles was investigated in two different neuroblastoma cell lines. The anti-cancer effects of the drug-loaded nanoparticles were increased in comparison to doxorubicin solution. Based on these result a standard protocol for the preparation of doxorubicin-loaded HSA nanoparticles for further antitumoural studies was established.

Keywords: Nanoparticles; Doxorubicin; Human serum albumin (HSA); Physicochemical characterisation; Cell viability

1. Introduction

Doxorubicin is a widely used antineoplastic agent. However, its use is limited by its high toxicity, especially its cardiotoxicity (Singal et al., 2000; Minotti et al., 2004).

A promising approach to increase the efficacy and to lower side-effects of antineoplastic drugs is their binding to drug delivery systems such as nanoparticles. Doxorubicin has been bound to nanoparticles made of different materials, as for example, poly(butyl cyanoacrylate) (PBCA) (Gulyaev et al., 1999; Steiniger et al., 2004), poly(isohexyl cyanoacrylate) (PIHCA) (Cuvier et al., 1992), poly(lactic-co-glycolic acid) (PLGA) (Yoo et al., 1999), and gelatin (Leo et al., 1997; Leo et al., 1999). In comparison to doxorubicin solutions the nanoparticle formulations showed several benefits: with particle sizes in the range of 100–500 nm a passive tumour targeting was possible due to the so-called enhanced permeation and retention effect (EPR effect) (Maeda et al., 2000). Furthermore, some of these colloidal carriers were able to overcome multidrug resistance in various cancer cell lines. For instance, Barraud et al. (2005) showed a 4.5-fold reduction of the IC_{50} (half maximal inhibitory concentration) in HepaRG cells with PIHCA-nanoparticles compared to free doxorubicin. Doxorubicin-loaded PBCA nanoparticles coated with polysorbate 80 were able to transport the drug into the brain of glioblastoma-bearing rats (Steiniger et al., 2004; Ambruosi et al., 2006). This effect resulted in an increased survival time and in long time remissions of more than 20% of the treated animals compared to control.

In contrast to these nanoparticulate systems, human serum albumin (HSA) nanoparticles offer the benefits to carry functional groups (i.e. amino and carboxylic groups) that can be used for surface modifications (Wartlick et al., 2004a). Additionally, it has to be expected that HSA based nanoparticles will be well tolerated without any serious side-effects, which is supported by clinical studies with registered HSA based particle formulations such as Albunex™ (Feinstein et al., 1990; Geny et al., 1993) and Abraxane™ (Damascelli et al., 2001; Ibrahim et al., 2002). For this reason, the drug loading capacity of doxorubicin to HSA nanoparticles was investigated. The HSA nanoparticles were prepared by a desolvation technique as

* Corresponding author. Tel.: +49 69 798 29692; fax: +49 69 798 29694.
E-mail address: k.langer@em.uni-frankfurt.de (K. Langer).

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described previously (Weber et al., 2000; Langer et al., 2003). Glutaraldehyde was used for the stabilisation of the nanoparticles. As shown before, the stability of HSA nanoparticles can be controlled by the amount of glutaraldehyde used (Weber et al., 2000; Wartlick et al., 2004b).

For drug loading of nanoparticles three major strategies can be employed: (1) covalent attachment of the drug to the particle surface (Eatock et al., 1999) or to the polymer prior to preparation (Yoo et al., 1999), (2) adsorption of the drug to a preformed carrier system, and (3) incorporation of the drug into the particle matrix during particle preparation. While the first possibility leads to a new chemical entity (NCE) (Duncan, 2003), the simple adsorption to a preformed carrier system bears the risk of drug loss by desorption processes. Therefore, a promising method appears to be drug incorporation into the particle matrix. Incorporation has been shown to protect pharmacological active substances from degradation during storage as well as from early degradation/inactivation after injection.

In the present study, doxorubicin was adsorbed to preformed HSA nanoparticles or was incorporated into the matrix of HSA nanoparticles, using a previously described desolvation method (Weber et al., 2000; Langer et al., 2003). The HSA nanoparticles were investigated for their anti-cancer activity in two neuroblastoma cell lines (UKF-NB3; IMR 32).

2. Materials and methods

2.1. Chemicals and reagents

Human serum albumin (HSA, fraction V, purity 96–99%, 65,000 Da) and glutaraldehyde 8% solution were obtained from Sigma (Steinheim, Germany). Doxorubicin hydrochloride was a gift of Sicor (Milan, Italy). Solvents such as ethanol, acetonitrile, and all other reagents were purchased from Merck (Darmstadt, Germany) and were of analytical grade.

Unless otherwise stated all of the experiments were performed in triplicate.

2.2. Adsorption isotherm of doxorubicin to HSA

In order to determine the adsorption isotherm of doxorubicin to HSA in solution, different amounts of doxorubicin (0.25 mg/ml, 0.5 mg/ml, 0.75 mg/ml, 1.0 mg/ml, 2.0 mg/ml, 4.0 mg/ml) were incubated in a 20 mg/ml HSA-solution under constant shaking (650 rpm, Thermomixer comfort, Eppendorf, Hamburg, Germany) for 2 h at 20 °C. After incubation, the solutions were centrifuged through Amicon Ultrafree-MC centrifugal filter devices with a Biomax®30 membrane (size exclusion 30,000 Da, Millipore, Bredford, USA) according to the supplier's instructions. The concentration of doxorubicin in the ultrafiltrates was determined by HPLC analysis. The fraction of doxorubicin adsorbed to HSA as well as the equilibrium concentration of doxorubicin in the solution was calculated and the adsorption isotherm was plotted.

Prior to the determination of the adsorption isotherm the suitability of the filter devices was assured. To assure that no unspecific adsorption of doxorubicin to the filter material takes place, drug solutions ranging from 0.5 mg/ml to 4.0 mg/ml were filtered, and the drug recovery was measured by HPLC. The retention of HSA by the filter was examined by filtration of a 20 mg/ml HSA solution followed by protein determination in the filtrate by standard BCA assay (Pierce, Rockford, USA) (Smith, 1995).

2.3. Preparation of empty HSA nanoparticles

Empty HSA nanoparticles were prepared using a previously described desolvation technique (Weber et al., 2000; Langer et al., 2003). Specifically, 200 mg of HSA were dissolved in 2 ml of purified water and the pH was adjusted to 8.2 with 0.01 M NaOH. Under constant stirring (600 rpm) at room temperature 8 ml ethanol were added (1 ml/min) using a tubing pump (Ismatec IPN, Glattbrugg, Switzerland). Following the desolvation process the particles were stabilised by the addition of an aqueous 8% glutaraldehyde solution (1.175 μl/mg HSA). The crosslinking process was performed under stirring of the suspension over 24 h. The resulting nanoparticles were purified by repeated centrifugation (16,100 × *g*, 12 min, Centrifuge 5415D, Eppendorf, Hamburg, Germany) and redispersion in purified water by ultrasonication (Sonorex, Bandelin, Berlin, Germany) in order to eliminate excipients such as ethanol and glutaraldehyde.

2.4. Adsorption of doxorubicin to empty HSA nanoparticles

A stock solution of the doxorubicin (5 mg/ml) was prepared, and volumes between 50 μl and 800 μl were added to 20 mg of empty nanoparticles. The volume was adjusted with water to 4.0 ml. The mixture was stirred for 2 h (650 rpm) at room temperature to achieve an adsorption equilibrium of doxorubicin to the particle surface. The nanoparticles were washed as previously described. The supernatants of the washing steps were collected and the concentration of free doxorubicin was analysed by HPLC.

2.5. Incorporation of doxorubicin into HSA nanoparticles

According to the adsorption isotherm of doxorubicin to HSA in solution aliquots of 0.5–4.0 mg of doxorubicin and 20 mg of HSA were dissolved in 1 ml of purified water and stirred for 2 h to achieve adsorption of the drug to the protein. Nanoparticles were formed by addition of 3 ml of ethanol via a peristaltic pump (LKB, Bromma, Sweden) (1 ml/min) under constant stirring (650 rpm), and crosslinking was performed by addition of an aqueous 8% glutaraldehyde solution (0.294–1.175 μl/mg HSA, 24 h) as described above. The desolvation step was carried at pH 6.5 as well as after pH adjustment to 8.2. During particle purification the supernatants were collected, the drug content was measured by HPLC, and the loading efficiency of doxorubicin to the nanoparticles was calculated. The resulting particles were characterised with regard to size, size distribution, and zetapotential.

2.6. Measurement of particle size and zetapotential

The average particle size was measured by photon correlation spectroscopy (PCS) using a Malvern Zetasizer 3000HSA (Malvern Instruments Ltd., Malvern, UK). The samples were diluted 1:400 with purified water and were measured at a temperature of 25 °C at a scattering angle of 90°. The zetapotential was measured using the same instrument modified with a dip-cell (Malvern Instruments Ltd., Malvern, UK). For this analysis the nanoparticles were diluted 1:200 with 50 mM phosphate-puffer pH 7.4 and the zetapotential was determined by Laser Doppler microelectrophoresis.

2.7. Long-time stability of HSA nanoparticles with incorporated doxorubicin

Aqueous dispersions of nanoparticles with embedded doxorubicin were stored at 4 °C for a period of 1.5 years. After predetermined storage times, samples were taken, and the nanoparticles were redispersed by vortexing for 1 min (Vortex Genie, Scientific Industries Inc., NY, USA). As stability parameters particle size and polydispersity were determined as described above.

2.8. HPLC analysis of doxorubicin

A Hitachi D7000 HPLC system (Hitachi Ltd., Tokyo, Japan) with UV–vis and fluorescence detector was used for the quantification of doxorubicin in the samples. For the assay a reverse phase column (LiChroCART® 250-4 LiChrospher® 100 RP-18, Merck, Darmstadt, Germany) was used, and separation was obtained using an isocratic mixture of water and acetonitrile (70:30) containing 0.1% trifluoroacetic acid (Configliacchi et al., 1996). The flow rate was set to 0.8 ml/min, and the absorption (250 nm) and fluorescence (excitation 560 nm, emission 650 nm) were recorded. Under these conditions the retention time for doxorubicin was about 12 min. Doxorubicin concentrations were calculated relative to a calibration curve.

2.9. Cells

The N-myc amplified cell line UKF-NB-3 was established from metastases harvested in relapses in one of our patients with Evans stage 4 NB (Cinatl et al., 1992). IMR-32 cells were obtained from ATCC (Manassass, VA, USA). Both cell lines were propagated in IMDM supplemented with 10% FBS, 100 IU/ml penicillin, and 100 mg/ml streptomycin at 37 °C.

2.10. Cell viability-assay

Cell viability was investigated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye reduction assay after (Mosmann, 1983) modified as described before (Wartlick et al., 2004a). Briefly, 2×10^4 cells were seeded in 96-well plates and incubated with nanoparticles starting with a concentration of 100 ng/ml doxorubicin. After 5 days, 25 μl MTT reagent per well was added for 4 h. Thereafter, 100 μl SDS solution per well (20% SDS in a 1:1 DMF/H_2O solution) was added for further 20 h. Plates were read on a multiwell scanning spectrophotometer at a wavelength of 560 nm and a reference wavelength of 620 nm. Cell viability was determined as the relative reduction of the optical density which correlates with the amount of viable cells in relation to cell control. The inhibitory concentration of 50% (IC_{50}) was calculated as the concentration of drug yielding 50% of dye reduction compared to untreated control.

3. Results and discussion

Many different possibilities have been discussed and tested to diminish the toxicity, to modify the body distribution, and to increase the antitumour efficacy of antineoplastic drugs. Doxorubicin was linked to various molecules such as *N*-(2-hydroxypropyl)-methacrylamide (HPMA) copolymers via a biodegradable oligopeptide spacer (Seymour et al., 1990), to proteins (transferrin; albumin) via acid sensitive hydrazone linker (Kratz et al., 1998a,b), to bovine serum albumin (BSA) via glutaraldehyde (Ohkawa et al., 1993), and to antibodies (Hurwitz et al., 1975). In the case of HPMA-conjugates a higher circulation half-life and 100-fold lower initial peak levels of doxorubicin in heart tissue were observed. After further optimisation these conjugates have now entered the clinical phase I/II for the treatment of endocrine related cancers (Duncan et al., 2005). BSA-conjugates were able to overcome the multidrug resistance of a hepatoma rat cell line (AH66), and transferrin-conjugates showed antiproliferative activity in LXFL 529 human lung carcinoma cell line. A further promising approach to increase the efficacy and to lower side-effects of antineoplastic drugs is their binding to drug delivery systems such as nanoparticles.

The objective of the present study, therefore, was to optimise the drug loading of the cytostatic drug doxorubicin to HSA nanoparticles and to establish a standard protocol for their preparation. Two different ways of drug loading to the particle system were investigated: adsorption of doxorubicin to preformed nanoparticles as well as incorporation of the drug into the particle matrix.

Firstly, the adsorption of doxorubicin to HSA in solution as well as to preformed HSA nanoparticles was investigated. Prior to the adsorption experiments it was assured that no doxorubicin adsorbs to the used filter material and that no protein passes the filter. Under the conditions of the present study the recovery of doxorubicin within the investigated concentration range was approximately 100% (Fig. 1). No HSA was found in the ultrafiltrates (data not shown). To determine the adsorption of doxorubicin to dissolved HSA, doxorubicin was incubated with HSA for 2 h. Unbound drug was separated by ultrafiltration over 30 kDa size exclusion membranes. At doxorubicin concentrations between 0.25 mg/ml and 1.0 mg/ml more than 95% of the drug was adsorbed to albumin, while the adsorption decreased to about 75% at higher drug concentrations (Fig. 2). Different incubation times of 30 min and 4 h led to comparable binding efficiencies. Therefore, an incubation time of 2 h was predefined for further experiments. The resulting adsorption isotherm was analysed by fitting to the isotherms of Langmuir and Freundlich.

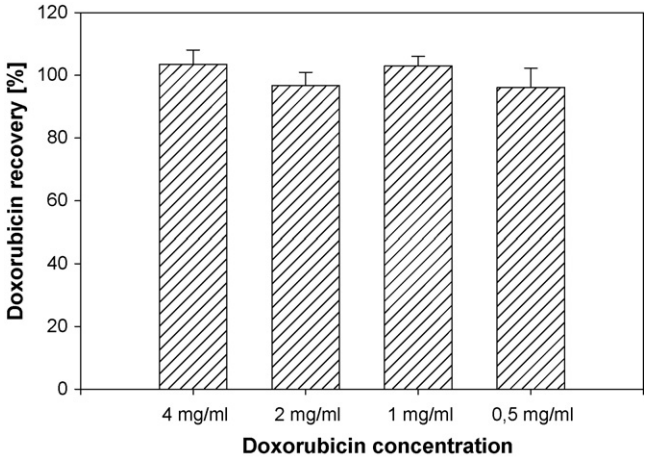

Fig. 1. Recovery of different doxorubicin concentrations filtered with Amicon Ultrafree MC Centrifugal Filter devices (mean ± S.D.; $n = 3$).

The best correlation was obtained after fitting to a Freundlich isotherm with a correlation coefficient of 0.98 in contrast to 0.86 for Langmuir. The adsorption experiment resulted in a maximum drug binding of 140 μg doxorubicin/mg HSA.

In addition, adsorption of doxorubicin to preformed nanoparticles was determined. Empty nanoparticles in a size of 157 ± 5 nm prepared by protein desolvation were incubated with doxorubicin for 2 h. At low doxorubicin concentrations, more than 95% (11.9–35.6 μg/mg) of the drug was adsorbed to the particles' surface (Fig. 3). The adsorbed drug fraction decreased with increasing concentrations of the drug, reaching a minimum of 69% (138 μg/mg) for 1.0 mg/ml doxorubicin. The fitting of these data also resulted in a Freundlich isotherm as mentioned before (correlation coefficient Freundlich 0.90; Langmuir 0.61). As expected, after adsorption no significant changes in particle size (158.5 ± 5.0 nm) were detectable, and the zetapotential was −31.9 ± 2.8 mV. Additionally, the stability of the adsorptive binding during the washing steps was examined. Less than 2% of the initial content was lost during each purification step. Even after 14 days of storage in water no considerable desorption of the drug was observed (data not shown).

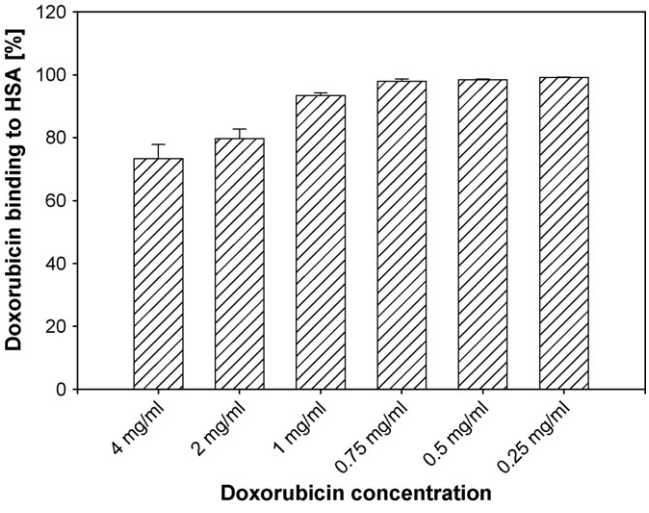

Fig. 2. Binding of doxorubicin to dissolved HSA: percentage of bound doxorubicin per 20.0 mg/ml of HSA in reference to the initial doxorubicin concentration (mean ± S.D.; $n = 3$).

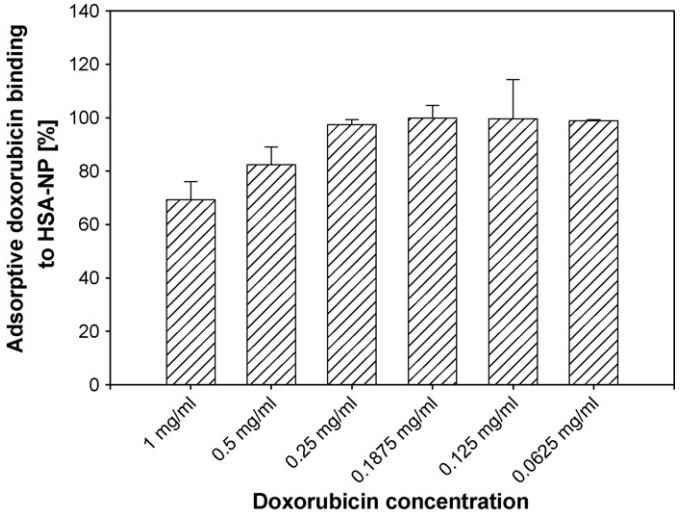

Fig. 3. Adsorptive binding of doxorubicin to 5.0 mg/ml of empty nanoparticles in reference to the initial doxorubicin concentration (mean ± S.D.; $n = 3$).

In the second part of the study, the incorporation of doxorubicin into the matrix of HSA nanoparticles was analysed. The incorporation procedure was based on the results obtained from adsorptive binding experiments of doxorubicin to dissolved HSA. Doxorubicin was adsorbed to HSA in solution prior to nanoparticle preparation by desolvation. Nanoparticles were prepared at two different pH values (pH 6.5 and 8.2) using four different doxorubicin concentrations in the range of 25–200 μg/mg. All preparations were stabilised with 1.175 μl glutaraldehyde 8%/mg HSA corresponding to 200% of the calculated amount necessary for the quantitative crosslinking of the 59 ε-amino groups of lysine in the HSA molecules of the particle matrix. Under the chosen preparation conditions incorporation efficiencies of up to 95% were achieved (Fig. 4). At concentration levels of 0.5–2.0 mg/ml doxorubicin, the nanoparticle preparation without pH adjustment (pH 6.5) led to an about 7%

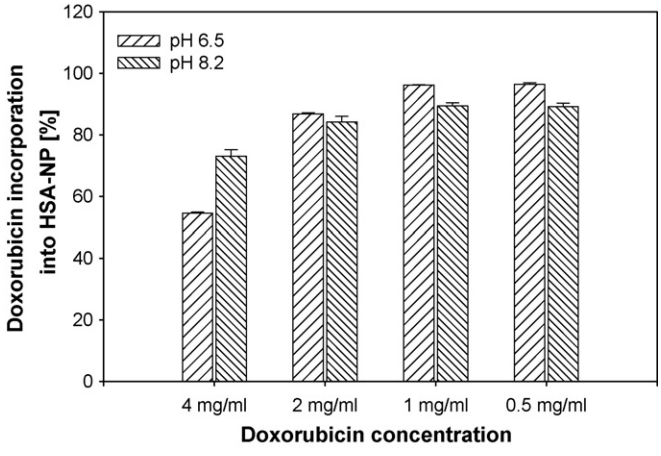

Fig. 4. Doxorubicin incorporation in HSA nanoparticles (20 mg/ml) in dependence on pH and doxorubicin concentration (mean ± S.D.; $n = 3$).

higher incorporation than preparations adjusted to pH 8.2. The reason for this may be explained by different HSA conformations at different pH values. The standard conformation "N" is present at neutral pH. A change to the "B" conformation occurs above pH 8 (Carter and Ho, 1994). This "B" conformation might have a slightly lower affinity to doxorubicin. In contrast, the drug loading efficiencies were reversed at higher doxorubicin concentrations (4 mg/ml). Without pH adjustment lower drug loading efficiencies were achieved than with preparations adjusted to pH 8.2. In this context, concentration-dependent drug effects on the secondary structure of HSA were shown for different drug molecules (Neault and Tajmir-Riahi, 1998; Purcell et al., 2000).

Doxorubicin concentration and pH influenced size and polydispersity of HSA nanoparticles. Monodisperse nanoparticles were obtained at doxorubicin concentrations of 0.5 mg/ml and 1.0 mg/ml without pH adjustment (Fig. 5). These particles showed a size of 300 ± 10 nm with a monomodal size distribution (polydispersity ≤0.1). Particle sizes increased, and broader size distributions (polydispersity 0.6–1.0) were observed with increasing drug concentrations. For example, concentrations of 4.0 mg/ml doxorubicin led to microparticles with a multimodal size distribution. This shows that doxorubicin influences the desolvation process during nanoparticle preparation. The doxorubicin hydrochloride, which was used in the experiments, dissociates in water into the protonized anthracycline and negatively charged chloride ions. Ionic compounds such as salts were previously shown to influence particle formation. The nanoparticle sizes slightly increase, probably due to the shielding of surface charges by the added ions and thus by a reduced repulsion between the macromolecular components during desolvation (Langer et al., 2003). For further experiments doxorubicin concentrations of 0.5 mg/ml and 1.0 mg/ml were chosen in combination with 20 mg/ml HSA. In order to prevent particle aggregation the preparation was performed in purified water without adjustment of the pH value.

To investigate the influence of the amount of the crosslinker glutaraldehyde on the doxorubicin incorporation efficiency and on the doxorubicin release during storage, nanoparticles were stabilised with 50%, 75%, 100%, and 200% glutaraldehyde,

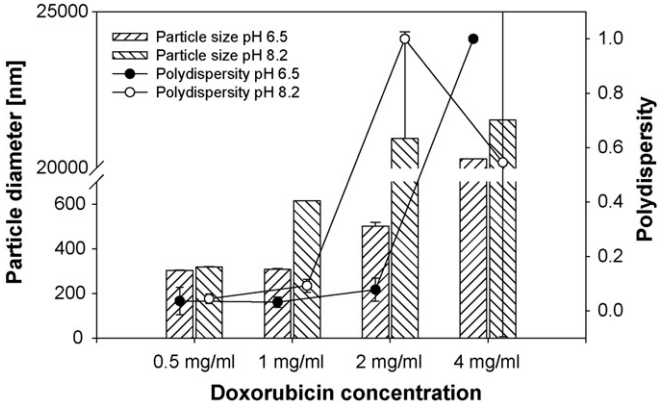

Fig. 5. Doxorubicin incorporation in HSA nanoparticles: influence of different amounts of doxorubicin and pH on size and polydispersity of the resulting nanoparticles (mean ± S.D.; $n = 3$).

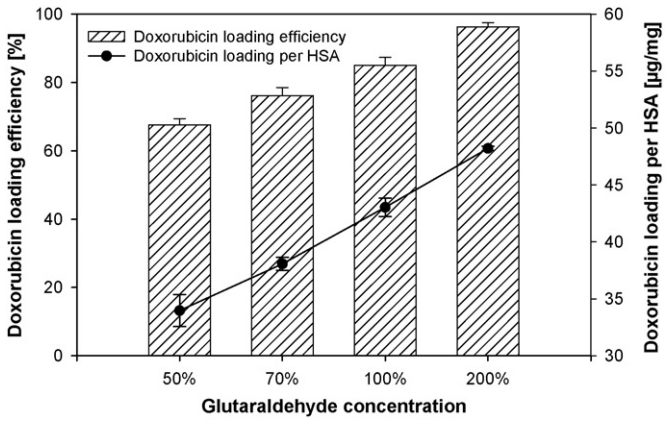

Fig. 6. Doxorubicin incorporation in HSA nanoparticles: relative [%] and absolute drug loading efficiency of doxorubicin [μg/mg] in dependence on percentage of glutaraldehyde used for particle stabilisation. Nanoparticles were prepared with 1.0 mg/ml doxorubicin and 20.0 mg/ml HSA (mean ± S.D.; $n = 6$).

respectively. It was hypothesised that higher glutaraldehyde concentrations should result in a more compact crosslinked matrix with smaller pores available for drug diffusion and, consequently, for drug release. The influence of the crosslinker was determined at a drug concentration of 1.0 mg/ml doxorubicin (Fig. 6). The highest incorporation efficiency of 96.3 ± 1.2%, corresponding to 48.2 ± 0.2 μg/mg, was achieved at 200% crosslinker concentration. In the case of nanoparticles crosslinked with 50% glutaraldehyde, the drug loading and entrapment efficiency decreased significantly to 67.6 ± 1.8% and 34.0 ± 1.4 μg/mg, respectively. At all crosslinker concentrations the particle size was in the range of 404 ± 14 nm, and the zetapotential was determined to be −56.1 ± 1.4 mV (Fig. 7). An explanation for the influence of the crosslinker on the drug loading efficiency may be the formation of covalent linkage between drug and matrix protein. Leo et al. (1997) showed that stabilisation of gelatin nanoparticles with glutaraldehyde resulted in covalent coupling of up to 70% of the used doxorubicin to the gelatin matrix of the particles. Similar effects cannot be ruled out for the proposed HSA nanoparticles. However, according to

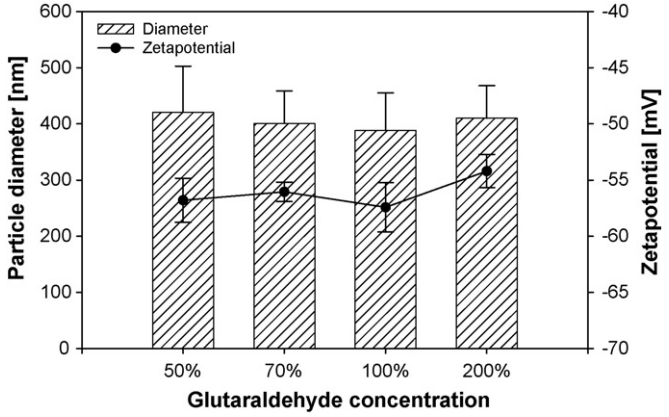

Fig. 7. Doxorubicin incorporation in HSA nanoparticles: influence of crosslinker amount used for particle stabilisation on particle size and zetapotential. Nanoparticles were prepared with 1.0 mg/ml doxorubicin and 20.0 mg/ml HSA (mean ± S.D.; $n = 6$).

our preparation method the ratio of amino groups in the HSA to amino groups in doxorubicin is significantly higher for the protein and, therefore, protein crosslinking by glutaraldehyde instead of doxorubicin conjugation is more probable. Experiments to prove this hypothesis are in progress.

Furthermore, the storage stability of the nanoparticle preparations was investigated. The doxorubicin-loaded nanoparticles were stored for up to 1.5 years in water at 4 °C, and at predefined times the samples were analysed with regard to size and polydispersity (Fig. 8). After 1 week of storage, the particles showed a uniform red sedimentation. However, the particles could easily be redispersed by shaking for 1 min. Over a storage period of 21 days the particle size decreased from 422 nm to 374 nm. After 6 months, the nanoparticles still showed an acceptable size just below 500 nm with a slightly increased polydispersity of 0.2. After storage periods of 1 and 1.5 years particles became difficult to redisperse, and sizes increased into the micrometer range with a multimodal size distribution. Therefore, long-term storage of the particles in suspension is not possible.

Finally, the effect of doxorubicin-loaded HSA nanoparticles on the viability of two neuroblastoma cell lines (UKF-NB3; IMR 32) was tested. Nanoparticles loaded with doxorubicin by adsorption (formulation A and B) as well as nanoparticles in which doxorubicin was incorporated (formulations C and D) were compared. For adsorptive drug loading 5 mg/ml HSA nanoparticles were incubated in the presence of 0.125 mg/ml (formulation A) and 0.250 mg/ml (formulation B) leading to drug loading efficiencies of 24.6 ± 0.3 μg/mg and 49.7 ± 0.5 μg/mg, respectively. For the preparation of nanoparticles with incorporated drug 20 mg/ml HSA were combined with 0.5 mg/ml (formulation C) and 1.0 mg/ml doxorubicin (formulation D). Glutaraldehyde concentrations ranging from 50% to 200% were used. The drug loading efficiencies varied between 17.1 ± 0.6 μg/mg and 24.3 ± 0.6 μg/mg for formulation C and between 33.5 ± 0.5 μg/mg and 48.4 ± 0.3 μg/mg for formulation D. For the cell culture experiment the nanoparticle samples were diluted with cell culture medium to predefined doxorubicin concentrations.

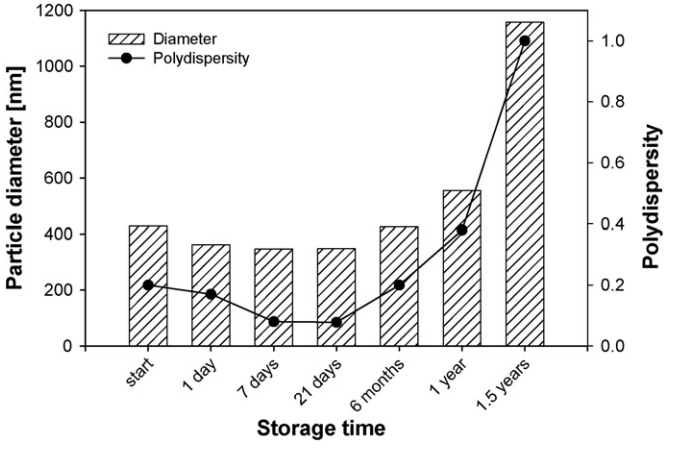

Fig. 8. Doxorubicin incorporation in HSA nanoparticles: influence of storage time on particle size and polydispersity. Nanoparticles were prepared with 1.0 mg/ml doxorubicin and 20.0 mg/ml HSA. Samples were vortexed for 1 min before measurement.

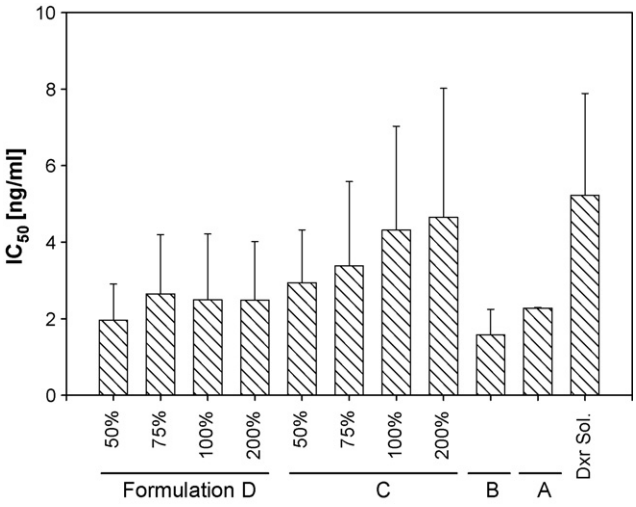

Fig. 9. Influence on cell viability (IC_{50} in ng/ml) of different doxorubicin-loaded HSA nanoparticle formulations compared to doxorubicin control solution in UKF-NB3 cell line: adsorptive bound doxorubicin (A and B) and nanoparticles with incorporated doxorubicin (C and D) stabilised with glutaraldehyde between 50% and 200% (mean ± S.D.; $n = 3$).

The cells were incubated for 5 days with the respective particle samples in concentrations of 0.75–100 ng/ml doxorubicin. The results of the performed MTT-assay for UKF-NB3 and IMR 32 cells are shown in Figs. 9 and 10, respectively. Generally, all of the doxorubicin-loaded nanoparticles were still effective and led to inhibition of cell viability in a low concentration range. Therefore, no loss of drug efficacy after binding to nanoparticles was observed. Furthermore, all of the nanoparticle formulations of doxorubicin showed the trend of being more effective than the respective doxorubicin control solution.

In the case of UKF-NB3 cells the nanoparticles prepared in the presence of 1.0 mg/ml doxorubicin were characterised by inhibitory concentrations (IC_{50}) of 1.96 ± 0.94 ng/ml to

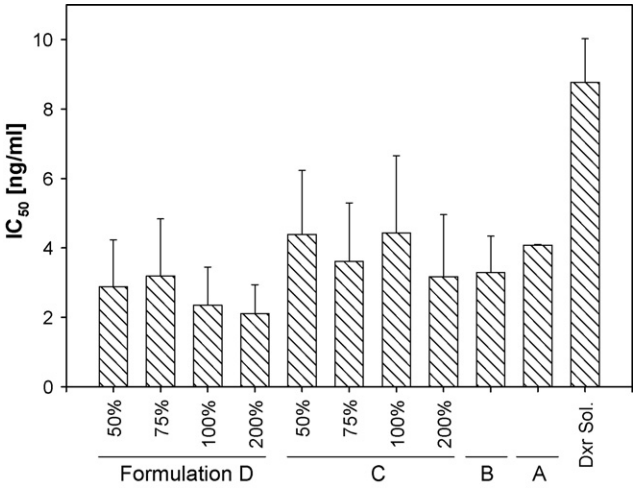

Fig. 10. Influence on cell viability (IC_{50} in ng/ml) of different doxorubicin-loaded HSA nanoparticle formulations compared to doxorubicin control solution in IMR 32 cell line: adsorptive bound doxorubicin (A and B) and nanoparticles with incorporated doxorubicin (C and D) stabilised with glutaraldehyde between 50% and 200% (mean ± S.D.; $n = 3$).

2.65 ± 1.55 ng/ml for nanoparticles with entrapped drug and of 1.58 ± 0.66 ng/ml to 2.28 ± 0.02 ng/ml for nanoparticles with adsorbed drug, respectively. The doxorubicin control solution showed an IC_{50} of 5.22 ± 2.66 ng/ml. Particle crosslinking after doxorubicin incorporation showed the trend that higher crosslinker concentrations led to higher inhibitory concentrations. For nanoparticles which were loaded with a doxorubicin concentration of 0.5 mg/ml the IC_{50} values increased from particles stabilised with 50% glutaraldehyde (2.94 ± 1.38 ng/ml) to 200% crosslinked nanoparticles (4.65 ± 3.37 ng/ml). Consequently, intracellular drug release may be decreased with nanoparticles crosslinked by higher glutaraldehyde concentrations. Therefore, a lower degree of particle stabilisation seems to be advantageous. Looking at the effect of drug loading it was observed that nanoparticles with a higher payload of doxorubicin showed an increased inhibition of cell viability, possibly because more drug is transported into the cell per single carrier.

Similar results were observed in IMR 32 cells (Fig. 10). All of the IC_{50} values of the particle preparations (2.11 ± 0.83 ng/ml to 4.42 ± 2.23 ng/ml) were significantly lower ($p \leq 0.001$) compared to the doxorubicin control solution (8.78 ± 1.25 ng/ml). As previously observed for UKF-NB3 cells, the same trend of higher doxorubicin loading resulting in a more pronounced inhibition of cell growth was detected. Interestingly, no clear correlation between crosslinking of the particle matrix and growth inhibition could be observed in IMR-32 cells. The reason for this difference remains unclear. Differences in uptake and degradation of HSA nanoparticles between the cell lines might be an explanation.

4. Conclusion

In our study we investigated the preparation of doxorubicin-loaded HSA nanoparticles. Doxorubicin may either be adsorbed onto the particle surface or incorporated into the particle matrix during particle preparation. In order to verify the influence on cell viability of doxorubicin after drug loading to the carrier system the 50% inhibitory concentration (IC_{50}) of the formulations was investigated in two neuroblastoma cell lines.

The adsorption study resulted in nanoparticles with a loading efficiency of more than 95% for initial doxorubicin concentrations equal or below 0.25 mg/ml. Particle sizes of the drug-loaded particles did not differ significantly compared to empty nanoparticles.

Nanoparticles with incorporated doxorubicin were prepared by incubating the drug with matrix protein prior to the desolvation and crosslinking step. The characteristics of the resulting nanoparticles were dependent on the concentration of doxorubicin, the amount of the crosslinker glutaraldehyde, and the pH value during the desolvation process. The best nanoparticles were obtained at doxorubicin concentrations $\leq$1.0 mg/ml with 20.0 mg/ml HSA, 100% crosslinking and no pH adjustment (pH 6.5). A stability study revealed that HSA nanoparticles prepared according to these standard conditions showed no changes in particle size even when stored in aqueous suspension for up to 6 months at 4 °C. Additionally, doxorubicin loading to the carrier system did not result in a reduced biological activity; inhibition of cell growth was comparable or even better when compared to a doxorubicin control solution.

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